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Project Summary

Application of Chemical Fractionation/Aquatic Bioassay Procedure to Hazardous Waste Site Monitoring

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The chemical fractionation/aquatic bioassay test basically involves biological testing, first using a given collection of leachate, surface water, or liquid waste, and then using fractions and subfractions of the original sample material. The final test result, derived from a compilation of these different bioassay responses, is used to identify bioactive fractions of the original sample material, to assess some of the additive, synergistic and/or antagonistic effects caused by the component waste chemicals, and to provide a preliminary (or screening) hazard evaluation for the aquatic ecosystem. While chemical analysis of sample material is not a part of the procedure, a combination of biological test data and chemical analytical data will allow for the identification of compounds and groups of compounds that present the greatest environmental hazard.

While the procedure has been successfully used for monitoring industrial pollutants, the overall technique was not considered to be ready for use at hazardous waste sites. Preliminary testing had indicated some potential problems with the chemical fractionation phase; therefore, an evaluation was conducted using a laboratory prepared waste leachate sample. Since the results from this initial evaluation indicated that procedural revisions were necessary, a series of experiments were then conducted to improve the chemical fractionation phase. When these procedural revisions had been made, another evaluation was conducted using samples taken from actual hazardous waste sites.

In spite of the complex matrix encountered when using liquid waste material, the fractionation technique was reasonably effective at partitioning the neutral organics. Partitioning of inorganics was also reasonably efficient. However, partitioning of polar organics was not particularly impressive, and several of the alcohols and acids were recovered in both inorganic and organic fractions. Further studies will perhaps improve the fractionation efficiency, and therefore improve the overall procedure's usefulness as a monitoring method.

This Project Summary was developed by EPA's Environmental Monitoring Systems Laboratory, Las Vegas, NV, to announce key findings of the research project that is fully documented in a separate report of the same title (see Project Report ordering information at back).

Introduction

Assessing current and potential problems at uncontrolled waste sites has been very difficult. The problem of evaluating complex chemical mixtures rather than specific chemical compounds causes part of the difficulty. Another problem is that toxicity data and environmental transport data are limited for many of the waste compounds, especially those that are byproducts of organic synthesis as opposed to commercial products. Biological monitoring

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techniques can often be very useful when dealing with such problems, and the biological procedures are particularly effective when combined with chemical analysis of the sample material.

The chemical fractionation/aquatic bioassay procedure is used to identify bioactive fractions of a particular sample, to assess some of the additive, synergistic, and/or antagonistic effects caused by the component chemicals, and to provide a preliminary (or screening) hazard evaluation for the aquatic environment. The procedure basically involves biological testing, first using a given collection of leachate, surface water, or liquid waste, and then using chemical fractions and subfractions of the original sample material. The final test result is derived from a compilation of these different bioassay responses. *Skeletonema costatum* (saltwater algae), *Selenastrum capricornutum* (freshwater algae), *Mysidopsis bahia* (saltwater crustacean), and *Daphnia magna* (freshwater crustacean), are used as test organisms during the assay. However, the saltwater algae and saltwater crustaceans tests are not conducted if an inland location of a particular waste site precludes any possibility of estuary contamination. There is a good data base available for these frequently used biological procedures that indicate assay responsiveness to many pollutant compounds, and some progress has been made toward method standardization.

In spite of the procedure's successful application for monitoring industrial pollutants, the overall technique was not considered to be ready for use at hazardous waste sites. Preliminary testing had indicated some potential problems with the chemical fractionation phase; therefore, an evaluation was conducted using a synthetic (laboratory prepared) waste leachate sample. Since the results from this initial evaluation indicated that procedural revisions were necessary, a series of experiments were conducted to improve the chemical fractionation phase of the overall technique. When these procedural revisions had been made, another evaluation was conducted using samples taken from actual hazardous waste sites.

Chemical Fractionation/Aquatic Bioassay Procedure

During each separate analysis, a portion of the original sample material is first tested for toxicity using the different bioassay procedures. If a toxic response (to include either stimulation or inhibition of the algal populations) does not occur in any of the component assays, no further testing is conducted using this particular waste or leachate collection. If a toxic response does occur for any of the component tests, a second portion of the waste material is then chemically fractionated, and each fraction is separately tested using the crustacean and algal assays. The resulting fractions or test samples are the (1) total organics, (2) base/neutrals, (3) organic acids, (4) organic residuals, (5) recombination of 2, 3, and 4, (6) total inorganics, (7) cations, and (8) anions. The fractionation procedure can process up to a 2-liter sample, but additional fractionations are frequently necessary before biological testing begins. While replicate fractionation runs are being completed, those fractions and subfractions collected from the initial fractionation are stored at approximately 4°C prior to compositing the respective final samples. When the fractionation phase is complete, each of the final test samples mentioned above should contain a sufficient volume (i.e., volume/concentration) to provide sample material for the four bioassay tests. Obviously, it is better to have an excess of sample material than to discover that there is insufficient material to complete the component assays.

The sample is initially filtered through a 0.45 μ glass fiber filter. The filtrate is then loaded on a resin column. An XAD-4 and an XAD-8 resin column are used in tandem with the eluate from the XAD-4 resin being passed through the XAD-8 column. The aqueous eluate that passes through the resin columns, and the additional water (HPLC grade) that is added to remove any remaining inorganics, are combined and designated as the total inorganic fraction. Organic compounds are eluted from the XAD-4 and XAD-8 resins with acetone and diethylether respectively.

The total organics fraction is concentrated (to remove the acetone and diethylether) and is then extracted with methylene chloride at a pH > 12 to isolate the base/neutrals. The remaining aqueous phase is extracted with methylene chloride at a pH < 1 to isolate the organic acids. A solvent exchange step (dimethylsulfoxide) completes the fractionation process for these subsamples. The remaining aqueous phase (i.e., organic residuals subfraction) is concentrated to dryness and resuspended in dimethylsulfoxide. A proportionate amount of base/neutrals, organic acids, and organic residuals are combined for the recombination fraction. This reconstituted total organics fraction provides a separate sample for biological testing.

The aqueous phase from the initial column separation (XAD-4 and XAD-8 resins) contains the total inorganics fraction. Separate subsamples of the inorganic fraction are then fractionated, using ion exchange resins, to provide the respective cation and anion samples. A Dowex 1-X8 column is used to provide the cation subfraction and a Dowex 50 W-18 column is used to provide the anion subfraction. Both columns are eluted with deionized water.

Of the nine separate sample types (i.e., original sample, total organics, base/neutrals, organic acids, organic residuals, recombination of organic subfractions, total inorganics, cations, and anions), only the original material is used for biological testing prior to completing the fractionation phase. If multiple fractionations are required, these would be completed and the resulting fractions composited (with the previous runs) before any additional samples are biologically tested. Eight concentrations or dilutions of each fraction can be tested using each of the four component bioassays, i.e., 0.01, 0.1, 1.0, 10.0, 25, 50, 75, and 100 percent. However, fewer dilutions will frequently provide sufficient information, i.e., 0.01, 1.0, and 100 percent. The 100 percent or original concentration is based on the approximate concentration that existed in the original sample material. Dilution media must be prepared for the respective algal assays, and dilution water must be prepared for the crustacean tests. In some cases, the testing laboratory might not wish to return a sample to the original concentration and would instead biologically test the more concentrated fraction or subfraction.

The biological test species are all of ecological importance to the respective freshwater and estuary environments; they are available commercially, and stock populations or cultures are fairly easy to maintain at the testing laboratory. However, it should be emphasized that none of the biological assays are conducted as definitive tests in which range finding evaluations precede assays to characterize the concentration response curve. The four component

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assays are, in fact, being used as screening procedures to test samples of unknown chemical composition, and for each of the respective assays, a minimum number of test organisms is used. The crustacean assays are both conducted under static conditions where test solutions are not renewed during the testing period. The static test obviously requires less sample material than the flow-through procedure which is sometimes a critical factor when planning assays for a limited amount of fraction and subfraction sample material.

Procedure Evaluation

Preliminary Testing

The current demonstration used both hazardous waste site sample material and a synthetic (laboratory prepared) leachate which contained a limited number of compounds, i.e., 2, 4-dimethylphenol, cadmium sulfate, 4-chlorophenol, and triethanolamine. The synthetic material was obviously not as complex as the actual hazardous waste and was used as sample material during the first part of the evaluation. Chemical analysis of the resulting fractions and subfractions indicated a need for further testing and revision of the fractionation procedure, i.e., the individual compounds were not efficiently partitioned during fractionation. This need for protocol revision was also reflected in some of the bioassay test data which were, of course, acquired from testing the poorly partitioned fractions. This initial demonstration also indicated the need for some minor revisions to the bioassay portion of the protocol, especially in the instructions for preparing the various fraction samples immediately prior to biological testing (e.g., solvents, nutrient supplements, suggested amounts of sample material, number of dilutions, etc.). Several problems were encountered when preparing the laboratory cultures of *Skeletonema costatum*, and specific protocol revisions have been made for the required salts, metals, and vitamins used in the culture media.

Several preliminary studies were then conducted in an effort to improve the fractionation scheme prior to fractionating the waste material. These preliminary efforts included (1) a pretest fractionation of a waste site sample, (2) an attempt to separately elute the organic acids and base/neutrals from the XAD-4 resin by sequential elution, (3) a test where phenol and pentachlorophenol concentrations were determined in a modified mass balance study using two different waste sample volumes, (4) a study of compound recovery (at two different spiking concentrations) where fractionations using the XAD-4 resin were compared with fractionations using the XAD-4/XAD-8 resin column sequence, and (5) a brief study which examined potential compound losses during sample concentration.

A complete method protocol for the chemical fractionation/aquatic bioassay procedure is given in the project report. An outline of the fractionation procedure is shown in Figure 1. The project report protocol is the most complete version of the procedure and is one that includes (1) the revisions made following the synthetic sample fractionation and associated biological testing, and (2) the revisions made following the separate experiments mentioned above. However, based on the subsequent fractionations using actual hazardous waste site material, additional method revisions, beyond those already incorporated into the procedure, will probably be required before an efficient fractionation can be consistently achieved and before the overall technique can be considered for use in an operational monitoring network.

Fractionation of Waste Site Material

The hazardous waste material used during the evaluation was collected from two different waste sites. While known toxic and carcinogenic compounds were present in the waste material from both sites, these particular sites were selected (one in California and one in Oregon) because of the diversity of chemical compounds known to be present at each location. Material from the different locations was designated as collection A and collection B, respectively.

Tables 1 and 2 show the recovery of selected compounds that were identified in the initial waste samples. Computer programs (i.e., computer assisted GC/MS) confirmed the identification of many additional waste fraction and waste subfraction compounds, but these selected organics are presented mainly for illustrative purposes. Concentrations are given for the original sample material, the three organic subfractions, and for the total inorganics fraction. Approximate detection limits provided with each table have been based on the recovery of internal standards and have attempted to include some estimate of potential interference caused by the presence of many additional compounds. The selected compound results are, in most cases, extrapolated values based on the original 2 liter sample volume, i.e., corrections made for volume adjustments and subsampling that occurred during the fractionation process. Obviously, the organics present in the inorganic fraction represent a deficiency in the fractionation procedure. Tables 3 and 4 present the respective inorganic results acquired using inductively coupled argon plasma spectroscopy (ICAP).

Collection A contained fairly high concentrations of polar compounds, and, when the current fractionation procedure was followed, the overall recovery of these polar organics was not as efficient as the observed recovery of the base/neutrals. In addition, those polar compounds which were recovered from the XAD-4 and XAD-8 resins were frequently eluted with the aqueous phase into the total inorganic fraction (Figure 1). This failure to achieve the intended partitioning of component compounds would have caused obvious data interpretation problems had these collection A fractionation samples been biologically tested. A bioassay result for the total inorganic sample would actually have been a test response, not only to the total inorganics but also to many organic compounds. Correspondingly, a bioassay test result for the total organic fraction, or for any of the organic subfractions, would have been a test response to samples that did not contain many of the respective organics that were, in fact, present in the original material. Polar organics were also present in the collection B material, but they apparently did not overload the binding capacity of the resins, and consequently the compounds were eluted, for the most part, into the total organic fraction.

Conclusions

The chemical fractionation/aquatic bioassay procedure is designed to test samples of leachate, surface water, or liquid waste material such as might be encountered when monitoring at hazardous waste sites. The procedure has been successfully used to monitor industrial pollutants, and the current demonstration was conducted to test the procedure using a more complex waste sample. The demonstration indicated some areas in which the procedure seemed to be fairly effective, but it

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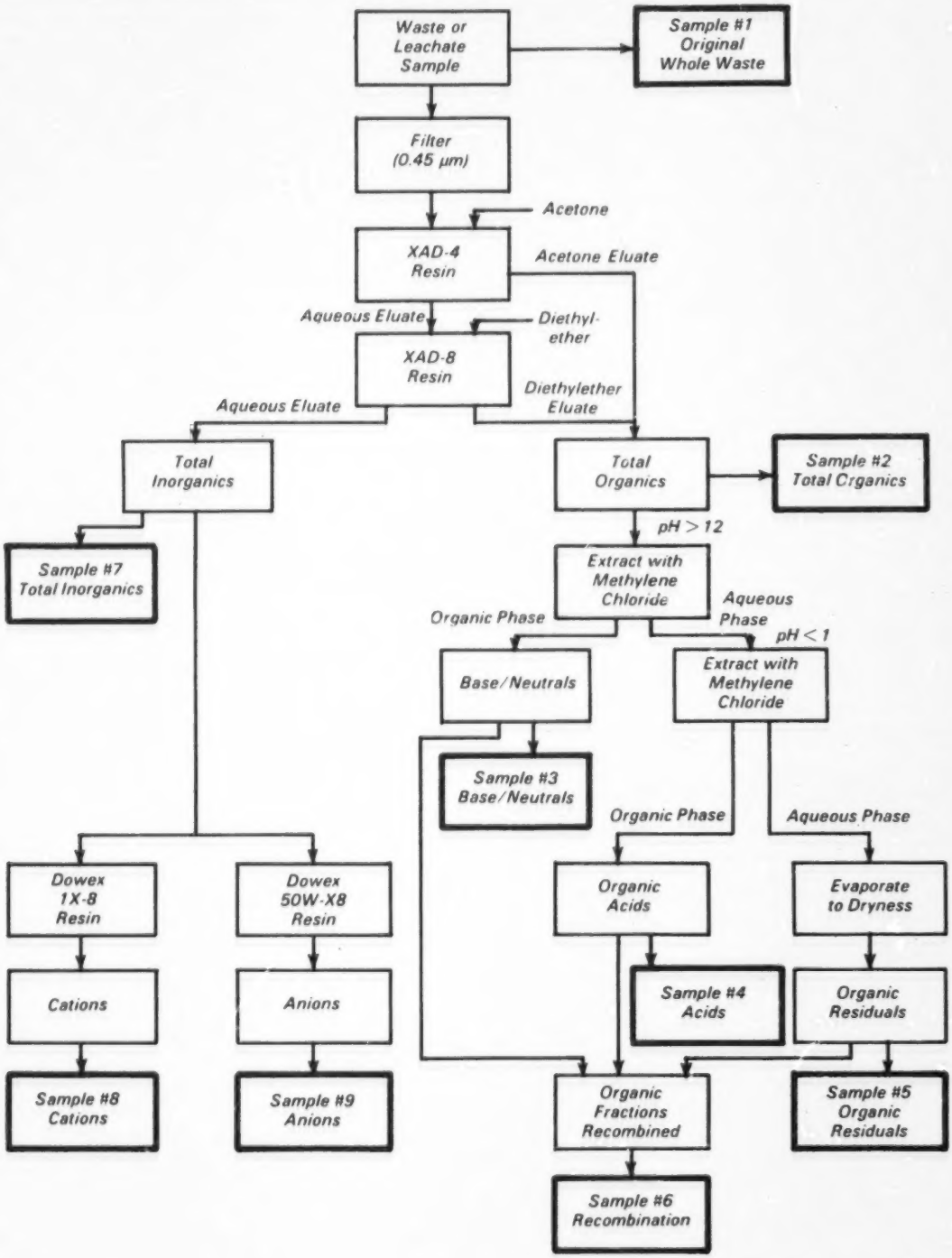

Figure 1. *Chemical fractionation phase of the chemical fractionation/aquatic bioassay procedure. A total of nine separate samples are ultimately provided for biological testing. The overall procedure was initially developed by G. E. Walsh and R. L. Garnas at the EPA laboratory in Gulf Breeze, Florida.*

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Table 1. *Recovery and Partitioning of Selected Organic Compounds Following Chemical Fractionation of Collection A Waste Site Material*

Compound	Whole Waste Concentration[1] (µg/l)	Whole Waste Estimated D.L.[2] (µg/l)	Base/Neutrals Concentration[1] (µg/l)	Base/Neutrals Estimated D.L.[2] (µg/l)	Organic Acids Concentration[1] (µg/l)	Organic Acids Estimated D.L.[2] (µg/l)
benzaldehyde	2,000	1000	740 (36)	50	170 (9)	20
benzoic acid	8,200	1000	ND	50	70 (1)	100
pentachlorophenol	20,000	1000	210 (1)	200	1,400 (7)	20
9,10-anthracenedione	16,000	1000	4,400 (26)	200	220 (1)	20
phenanthrene	ND	3000	1,900	200	140	20
anthracene	P	3000	2,500	200	180	20
carbazole	P	3000	2,100	200	50	20
biphenylene	ND	1000	1,200	50	70	20
fluoranthene	1,600	1000	1,400 (94)	50	110 (7)	20
pyrene	800	1000	950 (119)	50	70 (9)	20
p-phenylcarbanilic acid	28,000	1000	ND	50	ND	20
4-hydroxybenzene acetic acid	2,900	100	ND	50	ND	20
1,2-benzenedicarboxylic acid	360	100	ND	50	ND	20

Compound	Organic Residuals Concentration[1] (µg/l)	Organic Residuals Estimated D.L.[2] (µg/l)	Total Inorganics Concentration[1] (µg/l)	Total Inorganics Estimated D.L.[2] (µg/l)	Total Recovery (%)
benzaldehyde	350 (18)	NA	720 (34)	10	97
benzoic acid	700 (9)	NA	2,900 (36)	10	54
pentachlorophenol	ND	NA	7,200 (34)	10	42
9, 10-anthracenedione	2,900 (18)	NA	5,700 (34)	10	79
phenanthrene	NA	NA	ND	10	
anthracene	NA	NA	ND	10	
carbazole	NA	NA	ND	10	
biphenylene	NA	NA	ND	10	
fluoranthene	NA	NA	ND	10	101
pyrene	NA	NA	ND	10	128
p-phenylcarbanilic acid	2,700 (10)	NA	10,000 (36)	10	46
4-hydroxybenzene acetic acid	280 (10)	NA	1,000 (34)	10	44
1, 2-benzenedicarboxylic acid	420 (117)	NA	130 (36)	10	153

[1] - Calculated concentrations, given as µg/l, are based on a 2-liter total volume. The original extract concentrations have been multiplied by factors that correct for volume adjustments made during the fractionation. The percentage of compound recovered in a particular fraction is shown in parentheses.

[2] - Estimated detection limit: Estimate derived from GC/MS analysis of reference samples and from the respective volume adjustments that occurred during the extraction process.

ND - Not detected.

P - Present, but quantitative data not available due either to the presence of interfering compounds or to very low concentrations of the respective compound.

NA - Data not accessible.

Table 2. *Recovery and Partitioning of Selected Organic Compounds Following Chemical Fractionation of Collection B Waste Site Material*

Compound	Whole Waste Concentration[1] (µg/l)	Whole Waste Estimated D.L.[2] (µg/l)	Base/Neutrals Concentration[1] (µg/l)	Base/Neutrals Estimated D.L.[2] (µg/l)	Organic Acids Concentration[1] (µg/l)	Organic Acids Estimated D.L.[2] (µg/l)
pyridine	610	50	410 (67)	100	ND	100
2, 6-dimethylpyridine	530	50	74 (14)	100	ND	100
phenol	550	20	ND	100	720 (131)	100
quinoline	350	20	172 (49)	100	ND	100
benzaldehyde	P	1,000	ND	100	1100	100
2-methyl-2-hexanol	ND	100	ND	100	280	100
methyl pyridine	660	50	320 (48)	100	ND	100
1,2,3,4-tetrahydro quinoline	630	50	172 (27)	100	ND	100

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Table 2. *(Continued)*

Compound	Organic Residuals		Total Inorganics		Total Recovery (%)
	Concentration[1] (µg/l)	Estimated D.L.[2] (µg/l)	Concentration[1] (µg/l)	Estimated D.L.[2] (µg/l)	
pyridine	P	10	300 (49)	200	116
2,6-dimethylpyridine	ND	10	45 (8)	200	22
phenol	P	100	ND	200	131
quinoline	ND	10	ND	200	49
benzaldehyde	ND	10	ND	200	
2-methyl-2-hexanol	ND	100	110	200	
methyl pyridine	ND	10	370 (56)	200	104
1,2,3,4-tetrahydro quinoline	ND	10	ND	200	27

1 - Calculated concentrations, given as µg/l, are based on a 2-liter total volume. The original extract concentrations have been multiplied by factors that correct for volume adjustments made during the fractionation. The percentage of compound recovered in a particular fraction is shown in parentheses.

2 - Estimated detection limit: Estimate derived from GC/MS analysis of reference samples and from the respective volume adjustments that occurred during the extraction process.

ND - Not detected.

P - Present, but quantitative data not available due either to the presence of interfering compounds or to very low concentrations of the respective compound.

Table 3. *Inorganic Concentrations for Original Collection A Waste Material, Total Inorganics Fraction, Cation Subfraction, and Anion Subfraction (All concentrations given as mg/l.)*

	Original Waste Material (sample 1)	Total Inorganics Fraction		Cation Subfraction	Anion Subfraction	
		(sample 7)	% Recovery	(sample 8)	(sample 9)	% Recovery
Aluminum	420	130	80	140		27
Antimony	<15	<6	--	<1		--
Arsenic	<15	<6	--	<1		--
Barium	<7	<3	--	9.0		--
Beryllium	<1.5	<0.3	--	<0.05		--
Cadmium	14	4.7	87	9.8		57
Calcium	200	52	68	62		25
Chromium	440	140	83	450		84
Cobalt	130	41	82	84		53
Copper	710	190	70	410		47
Iron	470	160	89	570		100
Lead	3	<1.2	--	7.9		216
Magnesium	690	200	75	280		33
Manganese	38	11	75	16		35
Molybdenum	21	7.8	97	<0.2		--
Nickel	130	50	100	140		88
Selenium	<15	<6	--	<1		--
Silver	<15	<6	--	<1		--
Strontium	<1.5	<0.6	--	1.0		--
Thallium	20	6.6	86	<1		--
Titanium	<1.5	<0.6	--	<0.1		--
Vanadium	<7	<3	--	<0.5		--
Zinc	48	17	92	23		39
Fluoride	710	360	132		290	109
Chloride	14,000	4,500	84		3,400	65
Nitrate	31,000	11,000	92		8,000	69
Phosphate	<200	<200	--		<200	--
Sulfate	77,000	16,000	54		10,000	35
Nitrite	41,000	13,000	82		9,300	61
Cyanide	800	320	104		140	47

Note: Original extract concentrations have been multiplied by factors that attempt to correct for volume adjustments made during the fractionation. However, these corrections which allow for an estimate of element recovery (first in the total inorganic fraction and then in the cation and anion subfractions) have also artificially produced some recovery values that exceed 100 percent.

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Table 4. *Inorganic Concentrations for Original Collection B Waste Material, Total Inorganics Fraction, Cation Subfraction, and Anion Subfraction (All concentrations given as mg/l.)*

	Original Waste Material (sample 1)	Total Inorganics Fraction (sample 7)	Total Inorganics Fraction % Recovery	Cation Subfraction (sample 8)	Anion Subfraction (sample 9)	% Recovery
Aluminum	110	160	190	160		73
Antimony	<2	<10	--	<1		--
Arsenic	<2	<10	--	<1		--
Barium	<1	<5	--	0.5		--
Beryllium	<0.1	<0.5	--	<0.06		--
Cadmium	<0.5	<2	--	0.26		--
Chromium	0.4	0.44	83	0.4		50
Cobalt	0.4	<2	--	0.88		110
Copper	<0.5	<2	--	0.64		--
Iron	1,300	1,800	104	1,900		73
Lead	0.7	<2	--	41		--
Magnesium	200	320	120	340		85
Manganese	30	43	108	48		80
Molybdenum	<0.5	<2	--	0.6		--
Nickel	1	2	150	2.4		120
Selenium	<2	<10	--	<1		--
Silver	<2	<10	--	<1		--
Strontium	0.5	2.4	360	1		100
Thallium	<2	<10	--	<1		--
Titanium	<0.2	<1	--	<0.1		--
Vanadium	<1	<5	--	<0.5		--
Zinc	15	51	255	38		127
Fluoride	100	30	23		9	4.5
Chloride	420	1,300	232		170	20
Nitrate	<40	<40	--		<20	--
Phosphate	<40	<60	--		33	--
Sulfate	4,400	20,000	340		5,900	67
Nitrite	<20	<30	--		<10	--
Cyanide	0.02	0.06	225		0.008	20

Note: Original extract concentrations have been multiplied by factors that attempt to correct for volume adjustments made during the fractionation. However, these corrections which allow for an estimate of element recovery (first in the total inorganic fraction and then in the cation and anion subfractions) have aslo artificially produced some recovery values that exceed 100 percent.

also identified other areas where the procedure must be improved. In spite of a complex sample matrix, the fractionation technique was reasonably effective at partitioning the neutral organics. Partitioning of inorganics was also reasonably efficient. However, partitioning of polar organics was not particularly impressive, and several of the alcohols and acids were recovered in both inorganic and organic fractions. This breakthrough of polar organics was particularly dramatic when the original sample material contained high concentrations of these polar compounds. The individual bioassay tests should be capable of providing the required data if an efficient fractionation can be consistently achieved.

Based on the observation that polar organic compounds are frequently recovered in both organic and inorganic fractions, the chemical fractionation/aquatic bioassay procedure was judged as not being ready for use at actual hazardous waste sites, in spite of the fact that the overall procedure continues to be a potentially promising technique. Further studies will perhaps improve the fractionation efficiency and therefore improve the overall method applicability for use in a waste site monitoring effort.

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The complete report, entitled "Application of Chemical Fractionation/Aquatic Bioassay Procedure to Hazardous Waste Site Monitoring," (Order No. PB 86-109 493/AS; Cost: $16.95, subject to change) will be available only from:

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